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The Search for Biologically Active Secondary Metabolites*

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Abstract: Plants provide a rich source of novel biologically active compounds. Biological and chemical screenings are complementary approaches for the rapid detection and isolation of interesting new plant constituents. Biological screening followed by activity-guided fractionation has been used successfully in our laboratories for the discovery of antifungal, larvicidal and molluscicidal compounds. High performance liquid chromatography (HPLC) coupled to UV spectroscopy (LC/UV), mass spectrometry (LC/MS) and nuclear magnetic resonance (LC/NMR) has proved to be highly efficient for the chemical screening of crude plant extracts. In particular LC/MS and LC/MS/MS used with different ionisation techniques such as thermospray (TSP), continuous flow-FAB (CF-FAB) and electrospray (ES) have proved to be very efficient for the early recognition of molluscicidal saponins in *Swartzia madagascariensis* and *Phytolacca dodecandra*. The combination of LC/UV/NMR/MS was of great value for the investigation of polyphenols and bitter principles in Gentianaceae species. Among other examples, LC/NMR analysis of the antifungal crude extract of the African plant *Swertia calycina* is presented.

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1 INTRODUCTION

The plant kingdom represents an extraordinary reservoir of novel molecules. However, only a small percentage of the estimated 400 000–500 000 plant species around the globe has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even lower. Plants contain hundreds or thousands of metabolites and any phytochemical investigation of a given plant will reveal only a very narrow spectrum of its constituents. Furthermore, when a plant extract is screened for a specific activity, it will still have to be considered as 'uninvestigated' with respect to any other pharmacological activity. The potential of higher plants as sources for new drugs is thus still largely unexplored.[1]

When screening for biologically active plant constituents, the selection of the plant species to be studied is obviously a crucial factor for the ultimate success of the investigation. Besides random collection of plant material, targeted collection based on consideration of chemotaxonomic relationships and exploitation of ethnomedical information is currently performed. Plants used in traditional medicine are more likely to yield pharmacologically active compounds.

The process that leads from the plant to a pharmacologically active pure constituent is very long and tedious, and requires a multi-disciplinary collaboration

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of botanists, pharmacognosists, chemists, pharmacologists and toxicologists. As shown in Fig. 1 this approach involves different steps.[2]

In this paper the complementary role of chemical screening to the biological screening of pesticidal agents in crude extracts will be particularly emphasised.

2 BIOLOGICAL AND CHEMICAL SCREENING OF CRUDE EXTRACTS

Crucial to any investigation of plants with biological activities is the availability of suitable bioassays for monitoring the required effects.[2] In order to cope with the number of extracts and fractions from bioactivity-guided fractionation steps, the capacity for high sample throughput is necessary. The test systems should ideally be simple, rapid, reproducible and inexpensive. When deciding which bioassays to employ in research on plant constituents, the first step is to choose suitable screening organisms. In the case of pesticides, these are often insects, molluscs or fungi (Table 1).

2.1 Antifungal bioassays

The increasing incidence of mycoses associated with AIDS and also those arising after treatment by immunosuppressive drugs has given fresh impetus to the search for novel antifungal agents.

Bioautography combines TLC with a bioassay *in situ* and allows localisation of active constituents in a plant extract. Spore-producing fungi, such as *Aspergillus*, *Penicillium* and *Cladosporium* spp. in a nutrition medium are sprayed on the TLC plates after development.[3] After incubation, zones of inhibition appear where fungal growth is prevented by the active components of the plant extract.

Since direct bioautography is not possible with yeasts such as *Candida albicans* (Robin) Berkhout, a simple and rapid agar overlay assay has been developed.[4] This contact bioautography technique relies on the transfer of active compounds from the stationary phase into the agar layer (which contains the micro-organism) by a diffusion process. After incubation, the plate is sprayed with methylthiazolyltetrazolium chloride (MTT) which is converted into an MTT formazan dye by the fungus. Inhibition zones are observed as clear spots against a purple background.

In our laboratory, the use of these different techniques has permitted the successful isolation of a large number of fungicidal natural products.[5]

2.2 Larvicidal bioassays

Mosquitoes, in particular species of *Anopheles*, *Aedes* and *Culex*, are important vectors of tropical diseases: *Anopheles* spp. are responsible for the transmission of malaria which still remains endemic in more than 100 countries and affects 250 million people in the world; *Aedes* spp., in particular *A. aegypti* L, transmit diseases caused by arboviruses (*ar*thropod *bo*rne *virus*) such as yellow fever and dengue fever. The ideal method to control these diseases is the systematic treatment of the breeding places of the mosquito with larvicidal agents. A simple bench-top assay has recently been included in our screening assays and crude plant extracts are now systematically tested for larvicidal properties.[6] The testing procedure involves second-instar larvae of *A. aegypti*, which are exposed to various dilutions of the extracts, previously solubilised in dimethylsulfoxide (DMSO). Mortality is evaluated with the naked eye after 30 min and 24 h. A sample is considered active when all larvae have been killed after 24 h.

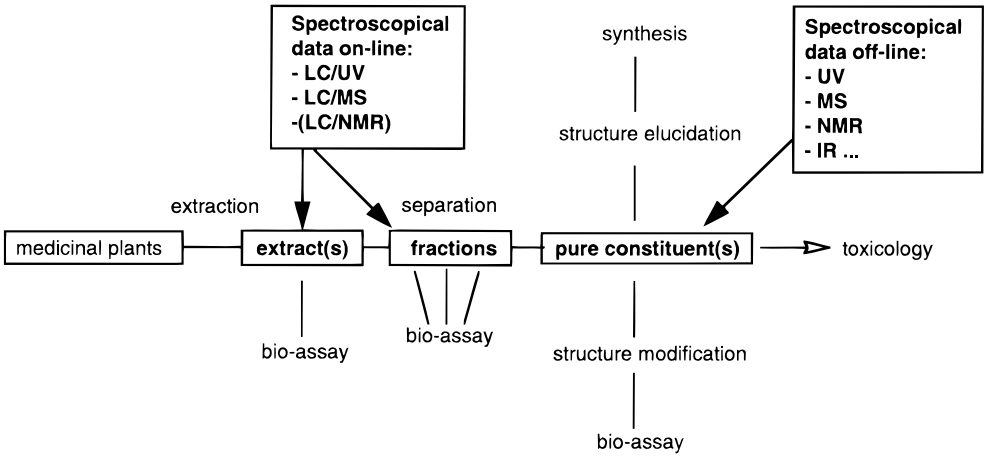


Fig. 1. Procedure for obtaining the active principles from plants and use of LC hyphenated techniques as strategic analytical screening tools during the isolation of constituents from a plant extract.

TABLE 1
Main Areas of Pesticide Research

Disease	*Activity*	*Screening organism*
Schistosomiasis	Molluscicidal	Aquatic snails: *Biomphalaria* spp. *Bulinus* spp.
Yellow fever Dengue fever	Larvicidal	Mosquito vectors: *Aedes aegypti*
Malaria	Larvicidal	Mosquito vectors: *Anopheles* spp.
Mycoses	Antifungal	Fungi: *Candida albicans* *Tricophyton* spp. *Mycrosporum* spp. etc.

2.3 Molluscicidal bioassays

Schistosomiasis, commonly known as bilharzia, is a parasitic disease caused by threadworms of the genus *Schistosoma* and is endemic throughout South America, Africa and the Far East. It affects more than 250 million people in over 76 countries. As shown in Fig. 2, the disease is spread in humans by a reproductive cycle of schistosomes which involves a stage implicating aquatic snails of the genera *Biomphalaria* and *Bulinus*.[7] One way to attack the problem of schistosomiasis is to destroy the carrier snails and thus remove a link in the life cycle. Growing molluscicidal plants in areas where schistosomiasis is endemic is a simple, inexpensive and appropriate technology for local control of the snail vector and could become a useful complement for the control of this disease in the future.[8] Schistosomiasis-transmitting snails of the species *Biomphalaria glabrata* (Say) are being used in our laboratories for screening plant extracts. The assay consists of exposing two snails to a solution containing the sample to be tested. Extracts are usually tested at an initial concentration of 400 mg litre^{-1}. Snails are examined after 24 h under a binocular microscope and mortality assessed by the absence of heart beats.

3 CHEMICAL SCREENING OF CRUDE EXTRACTS BY COMBINED LC/UV, LC/MS AND LC/NMR

As the number of targets for biological screening is limited, an efficient system for the chemical screening of the extracts is also needed in order to detect new leads which can potentially be interesting from a chemical viewpoint. Early recognition of plant metabolites, at the earliest possible stage of separation is also essential in order to avoid the time-consuming isolation of common constituents. Such a system requires hyphenated techniques which are able to provide efficient separation of the metabolites as well as good selectivity and sensitivity of detection and capacity to provide important structural information on-line.

3.1 Hyphenation in high performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is used routinely in phytochemistry to 'pilot' the preparative isolation of natural products (optimisation of the experimental conditions, checking of the different fractions throughout the separation) and to control the final purity of the isolated compounds.[9] HPLC is the most appropriate technique for an efficient separation of crude plant extracts and can be coupled with different spectroscopic detection methods.

In our laboratory, HPLC coupled with UV photodiode array detection (LC/UV-DAD) and mass spectrometry (LC/MS) have been used for the screening of crude plant extracts.[2,10,11] Recently, several trials have shown that HPLC coupled with nuclear magnetic resonance (LC/NMR) is also a very powerful complemen-

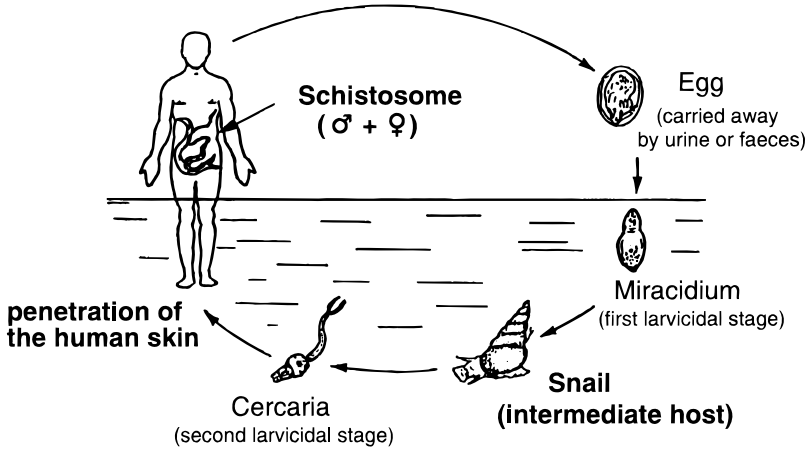


Fig. 2. Life cycle of *Schistosoma* species.

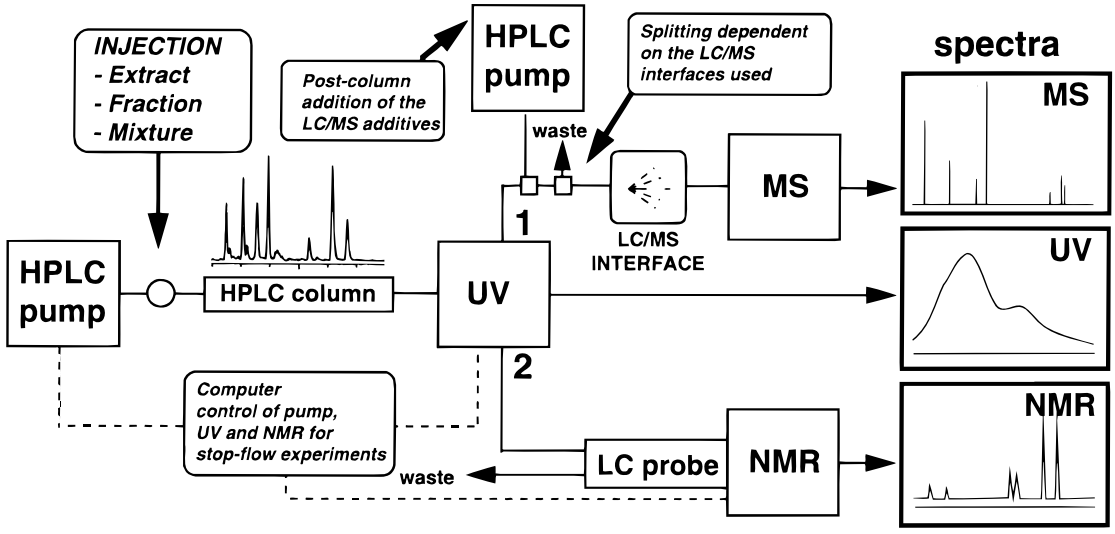


Fig. 3. Schematic representation of the experimental set-up used for (**1**) LC/UV/MS and (**2**) LC/UV/NMR analyses.

tary technique for the on-line plant metabolite identification.[12,13] This new tool is now being used to augment the existing LC/UV/MS configuration.[14]

3.2 Liquid chromatography/UV photodiode array (LC/DAD-UV)

HPLC coupled with UV photodiode array detection (LC/UV) has been used for more than a decade by phytochemists for screening extracts[15] and is now available in many laboratories. The UV spectra of natural products give useful information on the type of constituents and also, as in the case of polyphenols, information on the oxidation pattern. New instruments which allow the recording of UV spectra of reference compounds in databases and automatic computer matching are useful when screening for known constituents.

3.3 Liquid chromatography/mass spectrometry (LC/MS)

HPLC coupled to mass spectrometry (LC/MS) has been introduced recently and is still not widely spread in the phytochemical community.[10] At present, MS is one of the most sensitive methods of molecular analysis. Moreover, it has the potential to yield information on the molecular mass as well as on the structure of the analytes. Due to its high power of mass separation, very good selectivities can be obtained. The coupling between LC and MS has not been straightforward since the normal operating conditions of a mass spectrometer are very different from those use in HPLC.[16] To cope with this problem, many LC/MS interfaces have been built up. Each of them has its own characteristics and range of applications.[17] In our approach to LC/MS, used mainly for the HPLC screening of crude plant extracts, three interfaces, thermospray (TSP),[18] continuous flow FAB (CF-FAB)[19] and electrospray (ES)[20] have been investigated.[17] They cover the ionisation of relatively small non-polar products (aglycones, 200 uma) to highly polar molecules (glycosides, 2000 uma). LC/TSP-MS allows a satisfactory ionisation of moderately polar constituents such as polyphenols or terpenoids in the mass range of 200–800 uma. For larger polar molecules such as saponins (>800 uma) CF-FAB or ES are the methods of choice.[17] In our laboratory thermospray is the most widely used interface.

3.4 Liquid chromatography/nuclear magnetic resonance (LC/NMR)

HPLC coupled with nuclear magnetic resonance (LC/NMR), despite being known for over fifteen years, has not yet become a widely accepted technique, mainly because of its lack of sensitivity.[21] However, recent progress in pulse field gradients and solvent suppression, improvements in probe technology and the construction of high-field magnets have given a new impetus to this technique, which has important potential for on-line structure identification of natural products.[22] While the LC coupling itself is rather straightforward compared to LC/MS[23] (the samples are flowing in a non-rotating small cell having an internal volume which can vary from 60 to 180 μl, according to the probes, and which is connected at both ends with HPLC tubing), the main problem of LC/NMR has been the difficulty of observing analyte resonances in the presence of the much larger resonances of the mobile phase. This problem was aggravated in the case of typical LC reversed-phase operating conditions, where more than one protonated solvent was used and where the resonances changed frequencies during the change of solvent gradient. Furthermore, the continuous flow of

sample in the detector coil complicated solvent suppression. These problems have now been overcome thanks to the development of fast, reliable and powerful solvent suppression techniques such as WET,[24] which produce high-quality spectra in both on-flow and stop-flow modes. These techniques consist of a combination of pulsed field gradients, shaped rf pulses, shifted laminar pulses and selective ^{13}C decoupling and are much faster than the classical presaturation techniques previously used.[24] Thus, in reversed HPLC conditions, non-deuterated solvents such as methanol or acetonitrile can be used, while water is replaced by D_2O.

A general setup of the experimental configuration used for performing LC/UV/MS and LC/NMR analysis is presented in Fig. 3.

4 SEARCH FOR MOLLUSCICIDAL COMPOUNDS FROM PLANTS

4.1 LC/TSP-MS, LC/CF-FAB-MS and LC/ES-MS studies on the constituents of *Swartzia madagascariensis*

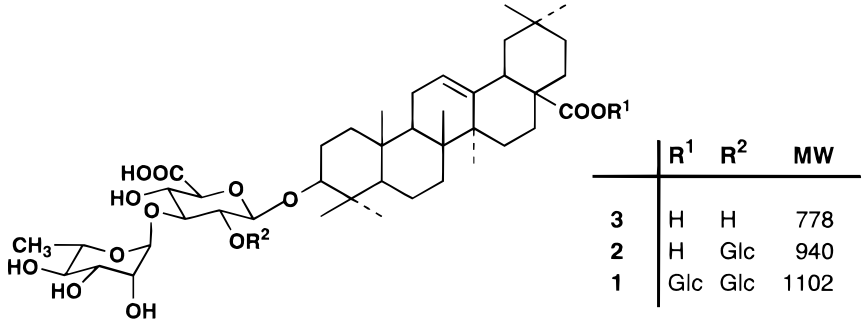
One of the most promising plants for the potential control of schistosomiasis-transmitting snails in Africa is *Swartzia madagascariensis* Devaux (Leguminosae). Aqueous extracts of the seed pods contain large amounts of saponins with high molluscicidal activity. These saponins have been characterised and field trials with extracts of the fruits have been performed in Tanzania.[25] The most active saponins are glycosides of oleanolic acid.

In order to have a rapid idea about the saponin content of a plant extract, LC/ES-MS was compared to LC/TSP-MS and LC/CF-FAB-MS for the analysis of saponins in the crude methanol extracts of the fruits of *S. madagascariensis*.[17] In order to discuss the LC/MS analysis of *S. madagascariensis* three oleanolic acid saponins **1, 2** and **3,** (Fig. 4) bearing respectively four, three and two sugar units were selected.

The LC/TSP-MS (Positive Ion (P.I.), NH_4OAc buffer) analysis of the extract exhibited the presence of triterpene glycosides derived from oleanolic acid (relative molecular mass: 456). Indeed, the TSP trace recorded at *m/z* 439 was characteristic for dehydrated oleanolic acid moieties $[A + H - H_2O]^+$ (trace, Fig. 5a). For **3,** a distinctive ion at *m/z* 796 and a fragment ion at *m/z* 650 were characteristic for a saponin bearing a diglycosidic moiety consisting of a terminal deoxyhexosyl unit (−146 uma) and a glucuronic acid (−176 uma) moiety. As rhamnose is the most frequent deoxyhexose occurring in saponins, it can be assumed from these on-line MS data that **3** was a saponin of oleanolic acid, substituted by a glucuronic acid unit and a rhamnosyl unit in the terminal position. The TSP spectra of saponins **1** and **2** were less clear than those of **3.** In both cases, characteristic signals for the oleanolic acid moiety were present, and fragment ions at *m/z* 795 were indicative of the presence of at least a glucuronic acid with a hexosyl unit. On the traces at *m/z* 941 and *m/z* 1103, no clear molecular ions for tri- or higher glycosylation were visible (trace, Fig. 5a). For these two metabolites, the LC/TSP-MS analysis alone could not give enough structural information on-line.

In the LC/CF-FAB-MS (Negative Ion (N.I.), glycerol matrix) analysis of the same extract (Fig. 5b), all the saponins exhibited intense deprotonated molecular ions $[M - H]^-$ and very weak ions characteristic for the aglycone moiety $[A - H]^-$ (*m/z* 455) and $[A - H - H_2O]^-$ (*m/z* 437). Furthermore, different characteristic cleavages were distinguishable. For **3,** ions at *m/z* 777 $[M - H]^-$, *m/z* 631 $[M - H - 146]^-$ and *m/z* 455 $[A - H]^-$ confirmed the results obtained with TSP. For **2,** an intense $[M - H]^-$ ion at *m/z* 939 was observed in the CF-FAB spectrum, showing that it was a triglycosylated saponin. The different fragment ions recorded in the CF-FAB spectrum of **2** (*m/z* 777 $[M - H - 162]^-$ and *m/z* 793 $[M - H - 146]^-$) confirmed that **2** was probably similar to **3** with one more hexosyl unit in position C-28 or branched at the diglycoside moiety. The CF-FAB spectra of **1** exhibited an intense molecular ion at *m/z* 1101 $[M - H]^-$ (see trace *m/z* 1101, Fig. 5b and spectrum, Fig. 6b). This indicated that **1** has one hexosyl unit (164 uma) more than **2.** Saponin **1** was thus a tetraglycosylated triterpene.

The LC/ES-MS analysis of the same extract (N.I., NH_4OAc buffer, CID 50 V) gave intense and clearly discernible $[M - H]^-$ ions and weak acetate anion adducts $[M + CH_3COO^-]$ for saponins **1**–**3** (trace, Fig. 5c, spectrum of **1,** Fig. 6c). With the aid of up front CID



	R^1	R^2	MW
3	H	H	778
2	H	Glc	940
1	Glc	Glc	1102

Fig. 4. Structures of selected saponins isolated from *Swartzia madagascariensis* (Leguminosae).

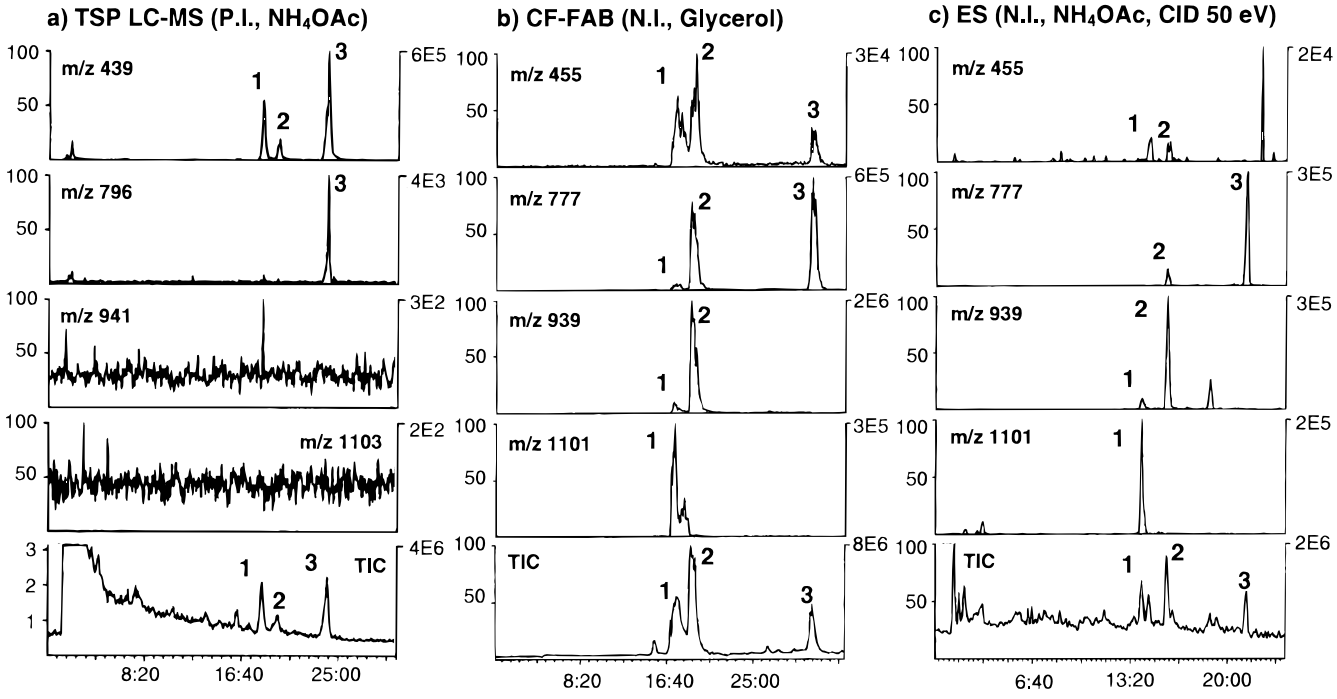


Fig. 5. Combined LC/TSP-MS (a), LC/CF-FAB-MS (b) and LC/ES-MS (c) of the methanol extract of *Swartzia madagascariensis* (Leguminosae). HPLC: C18 Nova-Pak (4 μm, 150 × 3·9 mm ID); gradient, acetonitrile : water (0·05% TFA) 30 : 70 — > 50 : 50 in 30 min (0·9 ml min^{-1}). The peaks identified correspond to the numbered structures in Fig. 4. P.I.: Positive ion mode, N.I.: Negative ion mode.

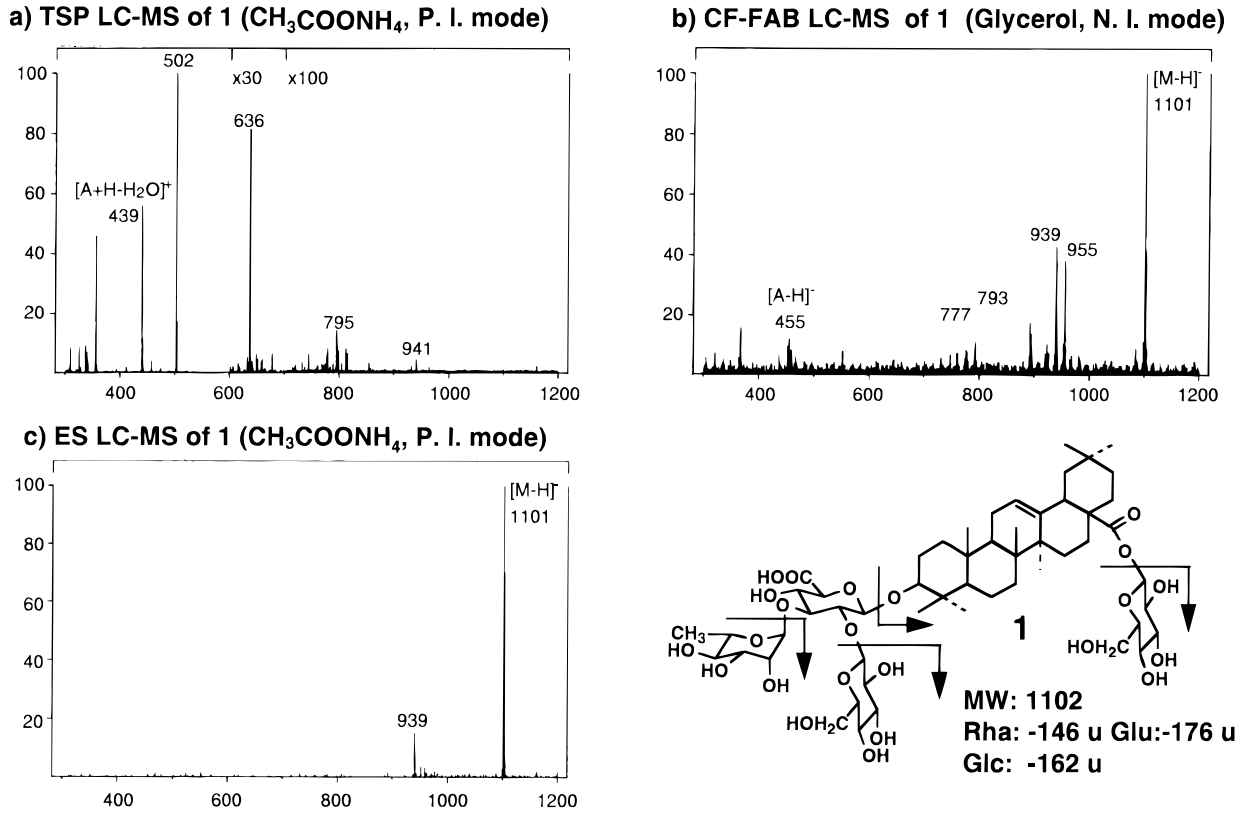


Fig. 6. Comparison of the LC/TSP-MS, LC/CF-FAB-MS and LC/ES-MS spectra of the tetraglycosylated saponin **1.** Same conditions as in Fig. 5.

in the entrance octapole of the ES source, ions due to the loss of different sugar moieties were also observable. Aglycone ions at *m/z* 455 appeared only for **3** and **2** but not for **1.** In ES, almost no peak broadening was observed producing clearly defined peaks in the selected ion traces (Fig. 5c). In CF-FAB, on the contrary, large diminution of the chromatographic resolution was due to the important splitting and the post-column addition of the glycerol matrix (Fig. 5b).

This example clearly shows the importance of the choice of the right interface for the ionisation of a given type of molecule. Conditions have to be chosen carefully according to the types of compound that have to be screened by LC/MS. LC/ES-MS gave mainly molecular ions but structural information can be obtained by the analysis of the subsequent fragmentation of these ions in MS/MS.

4.2 LC/MS^n studies on the constituents of *Phytolacca dodecandra*

The dried berries of endod, *Phytolacca dodecandra* L'Hérit (Phytolaccaceae), are used in Ethiopia as a soap substitute. The molluscicidal properties of their constituents were discovered by Lemma in 1965 and this plant rapidly became of great importance for the local control of schistosomiasis.[26] The sugar sequence of some of its saponins was tentatively identified on-line by LC-MS/MS. The LC/MS analysis of the methanolic extract of the berries was performed by LC/ES-MS in the negative ion mode with TFA as buffer. Under these conditions intense TFA molecular ion adducts $[M + CF_3COO^-]$ for the different saponins of the extract were observed (See spectrum of **4,** Fig. 7a).

For the determination of the sugar sequence of these saponins, an early prototype of a new ion-trap mass spectrometer was tested. With this instrument, it has been possible to perform not only an MS/MS experiment, but a multiple stage MS/MS experiment (MS^n). In MS^n, it was possible to isolate and excite only one ion of interest and thus to decrease the amount of consecutive reactions.[17] For example, on the pentaglycosylated saponin **4,** six stages of MS/MS were performed (MS^6). The sugar sequence information was obtained by successive decomposition of the main ion, as shown in Fig. 7.

The first step was the fragmentation of the strong TFA adduct at *m/z* 1363 (Fig. 7a), giving a deprotonated molecular ion $[M - H]^-$ (Fig. 7b). This latter ion yielded a first fragment at *m/z* 1087 (Fig. 7c). This first loss of 162 uma corresponded to the departure of the glucosyl moiety at position C-28. This sugar was particularly sensitive due to the ester linkage. The $[(M - Glc) - H]^-$ ion then cleaved into two fragments at *m/z* 941 and *m/z* 925 (Fig. 7d), showing the simultaneous loss of a rhamnosyl or a glucosyl unit, respectively. These losses were characteristic for a branched sugar chain. In Fig. 7e, the ion at *m/z* 779 issuing from the fragmentation of *m/z* 941 (− Rha) or *m/z* 925 (− Glc) was observed. This ion corresponded to the diglucoside moiety, which gave finally the mono-

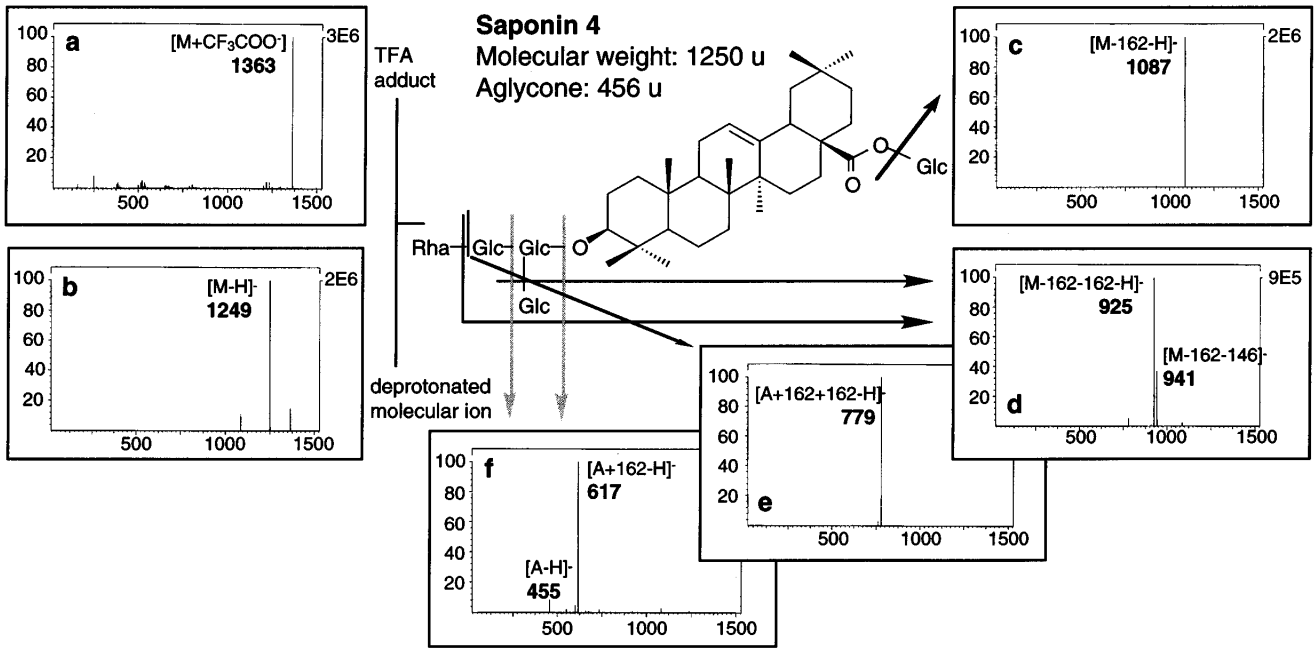


Fig. 7. ES MS^n spectra of the pentaglycosylated saponin **4** from the methanolic extract of *P. dodecandra* (Phytolaccaceae) fruits. Sample (1 mg ml^{-1}) injected by a syringe pump (5 μl min^{-1}) (infusion experiment). For LC/MS conditions, see Section 3.3. This experiment allowed a sequential fragmentation of the saponin sugar chain (cleavage of only one sugar at each MS/MS step), clarifying the structural determination.

glucoside $[(A + Glc) - H]^-$ at *m/z* 617 (Fig. 7f) and the aglycone ion characteristic of oleanolic acid at *m/z* 455.

Thus, this MS^n analysis showed that it was possible to cleave only one sugar at a time by adjusting the collision energy, making the interpretation simpler. This type of experiment was found to be very useful for clarifying the sugar sequence of saponins.

5 SEARCH FOR ANTIFUNGAL COMPOUNDS

5.1 LC/UV, LC/MS and LC/NMR studies on the constituents of *Swertia calycina*

The dichloromethane extract of *Swertia calycina* N. E. Br. (Gentianaceae), from Rwanda, presented a strong antifungal activity against *Cladosporium cucumerinum* Ell. & Arth. and *C. albicans*. According to the TLC assay, this activity was linked to a strong UV visible spot ($R_f = 0{\cdot}38$; petroleum ether + ethyl acetate, 1 + 1). As some xanthones are known to be antifungal agents,[27] one of these could be the active principle.

The LC/UV chromatogram of the dichloromethane extract of *S. calycina* was simpler than the methanolic extract and three main peaks (**5, 6** and **7**) were detected (Fig. 8). Compound **7** presented a UV spectrum with four absorption bands characteristic of a xanthone. Its TSP-MS spectrum exhibited a strong protonated ion at 303 uma, indicating a xanthone with a molecular weight of 302, thus substituted by one hydroxyl and three methoxyl groups. This information, together with the use of a home-made UV spectral library, permitted the identification of **7** as decussatin, a common xanthone in the Gentianaceae. The on-line data obtained for

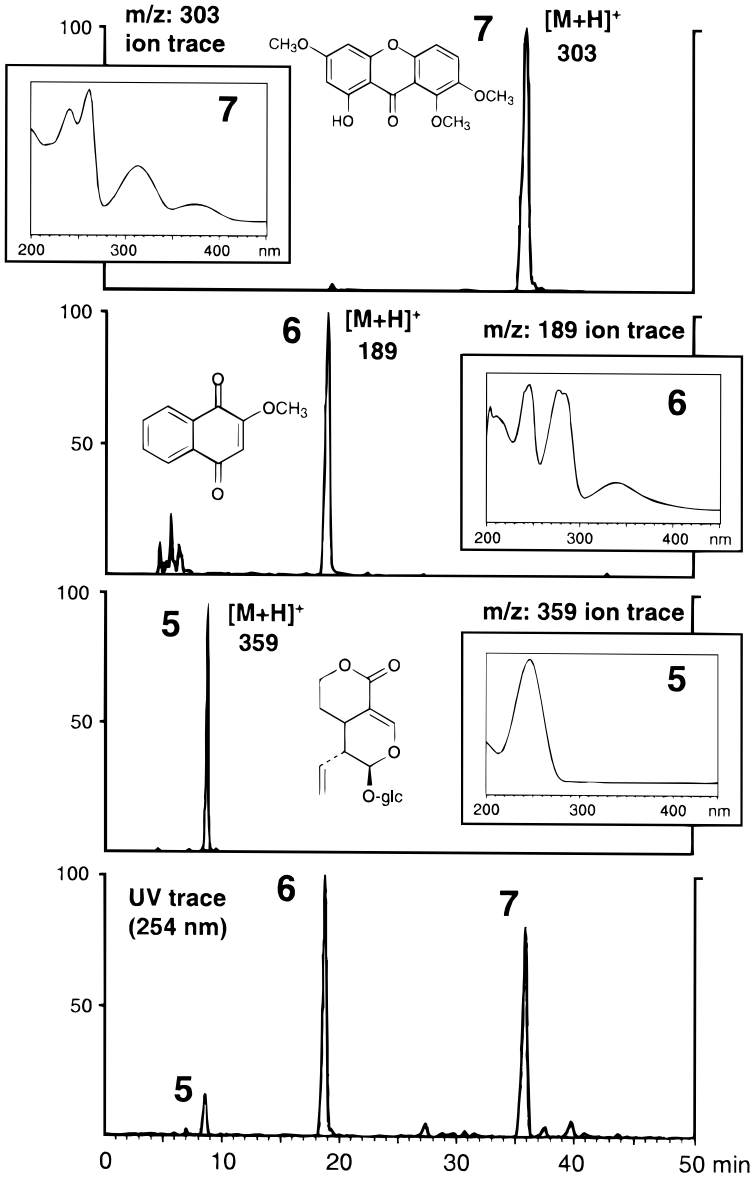


Fig. 8. LC/UV and LC/TSP-MS analysis of the crude dichloromethane extract of *Swertia calycina*. For each major peak, the single ion LC/MS traces of the protonated molecular ions $[M + H]^+$ are displayed, together with the UV spectra obtained on-line.

compound **5** indicated the presence of a secoiridoid-type molecule with a molecular mass of 358 uma. The loss of 162 uma observable in the LC/TSP-MS spectrum was characteristic for the presence of a hexosyl moiety. These data suggested strongly that **5** was most probably sweroside. The UV spectrum of **6** was not attributable to a common polyphenol of the Gentianaceae such as flavones or xanthones. It was very weakly ionised in TSP (see the background ions of the TSP spectrum in Fig. 10), but a protonated molecular ion was nevertheless found at *m/z* 189. This small molecular weight (188 uma) and the UV spectrum suggested that **6** could be a quinonic compound, but as no metabolite of this type had previously been found in Gentianaceae, it was not possible to identify it on-line.

In order to confirm the assignments and to obtain the more structural information on-line, the same extract of *S. calycina* was submitted to LC/[^{1}H]NMR analysis on a 500 MHz instrument.[14] The same LC conditions as for the LC/UV/MS analysis were used except that the water of the LC gradient system was replaced by D_2O. However the quantity of extract injected on to the column was increased to 1 mg to obtain at least 20 μg for each peak of interest.

For the suppression of the solvent signal of acetonitrile and its two ^{13}C satellites, as well as the residual HOD peak, a fast sequence called WET[24] was run before each acquisition. In the gradient LC run, the solvent peaks change frequency during the course of the experiment. As a result, the solvent suppression must be continuously adjusted for optimal performance. To do this, a one-transient-one-pulse experiment is used to find the solvent peaks prior to WET suppression (Scout Scan).[24] Thanks to this sequence, the transmitter is automatically adjusted to keep the biggest solvent peak at a constant frequency (acetonitrile), while the spectrometer is locked on D_2O.[14]

The on-line LC/NMR analysis of *S. calycina* provided [^{1}H]NMR spectra for all the major constituents. A plot of the retention time (*y* axis) versus the NMR shifts (*x* axis) permitted the localisation of the resonances of compounds **5, 6** and **7** (Fig. 9). On this unusual two-dimensional chromatogram, strong signals of aromatic methoxyl groups were observed around 4 ppm for **6** and **7.** Xanthone **7** exhibited two pairs of aromatic protons, while the quinonic compound **6** presented five other low-field protons. The more polar secoiridoid **5** showed different signals between 3 and 6 ppm. The important trace starting from 4·8 ppm (at 0 min) and ending to 4 ppm (at 30 min) was due to the change of the chemical shift of the residual negative water (HOD) signal during the LC gradient. The traces between 1 and 2·6 ppm were due to residual acetonitrile signal and solvent impurities.

A slicing of this bi-dimensional plot in single on-line LC/[^{1}H]NMR spectra for each constituent allowed a precise assignment of their specific resonances. Xanthone **7** exhibited three methoxyl groups at 3·92, 3·93 and 3·95 ppm. A pair of *meta*-coupled aromatic protons (1H, δ 6·55, *d*, J = 2·4, H-4) and (1H, δ 6·41, *d*, J = 2·4, H-2) was indicative of a 1,3-disubstituted A-ring. The B-ring protons exhibited a pair of *ortho*-coupled protons (1H, δ 7·36, *d*, J = 9·2, H-5) and (1H, δ 7·62, *d*, J = 9·2, H-6), suggesting a 1,3,5,6 or 1,3,7,8 substitution

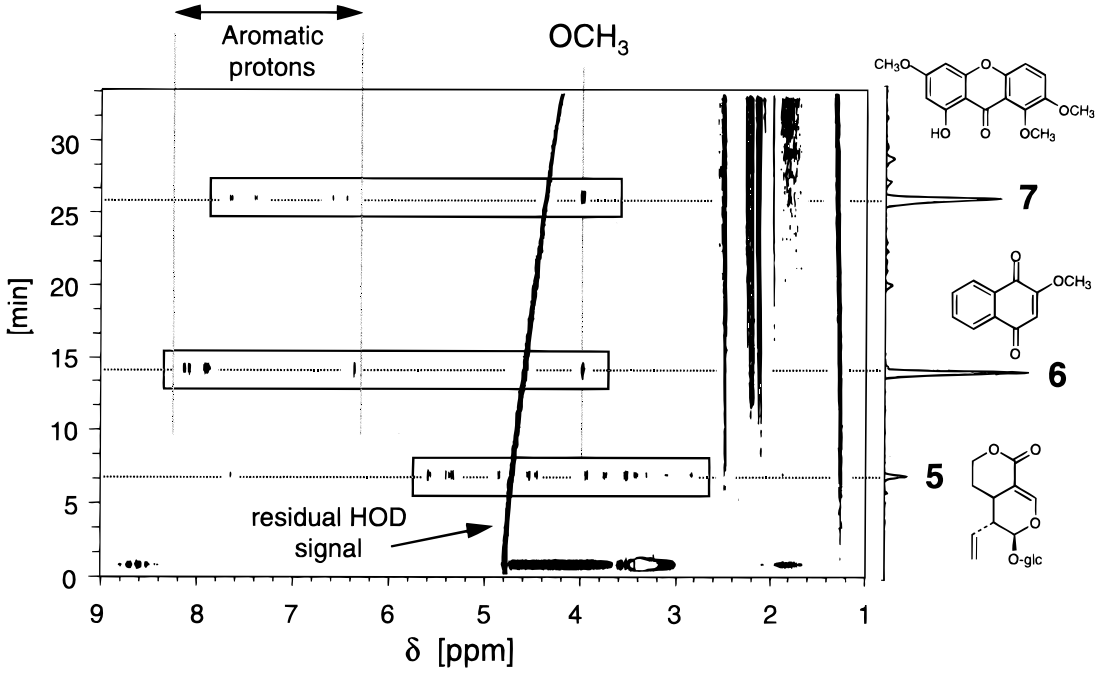


Fig. 9. Bidimensional LC/[^{1}H]NMR chromatogram of the crude dichloromethane extract of *Swertia calycina.* Methoxyl groups and aromatic proton signals of **6** and **7** are clearly visible together with all the resonances of the monoterpene glycoside **5.** The signal of HOD is negative and was continually shifted during the LC gradient.

pattern for **7**. A comparison of these NMR data together with UV and MS information allowed **7** to be identified as 1-hydroxy-3,7,8-trimethoxyxanthone (decussatin), as already suggested by the LC/UV/MS data.

On the LC/[^{1}H]NMR spectrum of **6** (Fig. 10), two signals (2H, δ 8·11, *m*, H-5,8 and 2H, δ 7·89, *m*, H-6,7) were characteristic of four adjacent protons of an aromatic ring with two equivalent substituents. The low field shift of the H-5,8 signal indicated that these two protons were in a *peri* position to the carbonyl functions, suggesting most probably the presence of a naphthoquinone nucleus.[28] The strong bands recorded in UV at 243, 248, 277 and 330 nm confirmed this deduction. The singlet at 4·35 ppm was attributed to H-3 and the remaining methoxyl group was thus at position C-2. With these on-line data and the molecular mass deduced from the LC/TSP-MS spectrum (188), **6** was finally identified as 2-methoxy-1,4-naphthoquinone (Fig. 9). As this was the first naphthoquinone to be reported in Gentianaceae, it was isolated and was found to be the compound responsible for the strong antifungal activity of the extract of *S. calycina*.[29]

This example showed that the LC/UV/MS and LC/NMR information obtained for *S. calycina* permitted a full structural identification of its main constituents.

6 CONCLUSIONS

Today the work of phytochemists is based mainly on the bioassay-guided fractionation of crude plant extracts. This type of approach has led to the isolation of numerous compounds with interesting activity.

Tropical plants are a rich source of molluscicidal, larvicidal and antifungal compounds. Testing of plant extracts for these properties is relatively straightforward and can be performed in a phytochemical laboratory with the necessary bioassays. Direct testing on schistosomiasis-transmitting snails or on yellow fever-transmitting mosquito larvae is performed for the establishment of molluscicidal or larvicidal activity and TLC bioautography can be used for ascertaining antifungal activity. However, before lengthy isolation work is undertaken unnecessarily, it is of great help to carry out chemical screening to avoid compounds of low interest. Newly developed on-line LC/MS, LC/UV and LC/NMR hyphenated techniques have rendered this operation possible within a short time-scale.

Different coupled mass spectrometric methods, such as TSP-MS, CF-FAB-MS, ES-MS and MS-MS, give important structural information about the triterpene glycosides present in plant extracts and can be used to identify, for example, known saponins present in the mixture. An efficiently targeted isolation of plant metab-

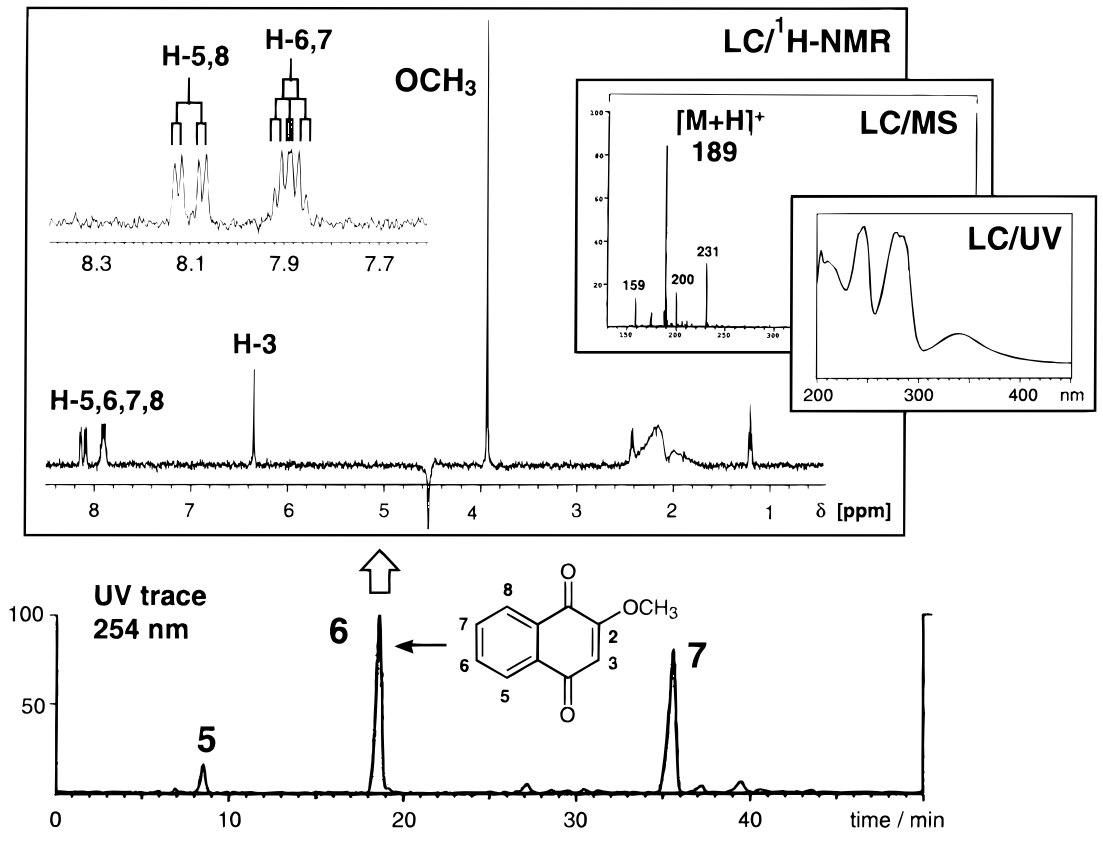


Fig. 10. Summary of all the spectroscopic data obtained on-line for the antifungal naphthoquinone **6** in the dichloromethane extract of *Swertia calycina*.

olites with potentially important bioactivities is thus possible.

In this field, the recent introduction of other hyphenated techniques such as LC/NMR[30] will render the on-line structural determination of metabolites more and more accurate and rapid. All these hyphenated techniques will provide a very efficient investigation tool for the phytochemist and allow the rapid chemical screening of a large number of crude plant extracts with a minute amount of material.

Natural products will continue to function as very important lead compounds. In the future, combination of simple biological assays enabling rapid screening of plant extracts with powerful hyphenated analytical techniques will provide, at an increasing rate, new active compounds with novel structures. The ultimate success of such a research will however depend on the effective collaboration between chemists, biologists and pharmacologists. Close cooperation between industry and universities is also essential for the efficient investigation and development of new leads. Only if such collaboration exists will promising molecules find an application one day as drugs or pest-control agents.

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